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(54) Title: A METHOD FOR OBTAINING 100 % MALE STERILE PLANTS TO SERVE AS THE FEMALE PARENT IN HYBRID SEEDS PRODUCTION

(57) Abstract: The invention relates to a method for obtaining seeds that yield 100 % male sterile plants to be used as the A-line for hybrid seeds production, comprising: a) selecting male sterile plants; b) inducing transient male fertility restoration (T-MFR) in said plants; c) self-pollination or crossing among plants that undergo T-MFR in step b and produced pollen, or cross-pollination between pollen produced in step b and any suitable male sterile plants; d) collecting the seeds of the fruits developed by the pollination of step c, said seeds will yield 100 % male sterile plants.

A METHOD FOR OBTAINING 100% MALE STERILE PLANTS TO SERVE AS THE FEMALE PARENT IN HYBRID SEEDS PRODUCTION

FIELD OF THE INVENTION

The present invention relates to a method for obtaining all female 100% male sterile plants for use as the female line in hybrid seeds production. More specifically the present invention relates to a method for obtaining seeds that yield 100% male sterile plants by manipulation of nuclear genes. Said seeds are obtained by self-pollination or cross pollination among male sterile plants that undergo transient male fertility restoration treatment (T-MFR) or by cross pollinating between the pollen produced by said plants and any suitable male sterile plants. The T-MFR according to the present invention is induced by subjecting the male sterile plants to controlled conditions of daytime and nighttime temperatures for a predetermined period of time. The present invention further relates to the pollen produced by the male sterile plants that undergo T-MFR, to the seeds which produce 100% male sterile plants, to the 100% male sterile plants developed thereof and to the male fertile F1 hybrid seeds produced by the crossing between said 100% male sterile plants and any other suitable male fertile C-line. The hybrid seeds produced according to the present invention yield fertile plants. The present invention specifically relates to said methods and seeds for plants of tomatoes and other species of the solanaceae family.

BACKGROUND OF THE INVENTION

Production of hybrid seed for commercial sale is a large developing industry and an extremely important one for the future feeding of the world population (Science, vol 283 pp. 310, 1999). Hybridization of plants is recognized as an important process for producing progeny having a unique combination of the desirable traits of the parental plant lines. Hybrid plants grown from hybrid seed benefit from the "hybrid vigour" of crossing two genetically distinct and carefully selected breeding lines. The agronomic performance of this progeny is superior to both parents, in vigour, yield, and uniformity. Thus, better performance of the hybrid seed varieties compared to open-pollinated varieties makes the hybrid seed more attractive to farmers. The F_1 hybrid plants cannot be practically duplicated since selfing or crossing of the F1 hybrid plants will cause segregation of the traits according to the Mandelian inheritance.

A plant is self-pollinated when pollen of a plant fertilizes the same flower or other flowers of the same plant. A plant is cross-pollinated if the pollen comes from a flower of a different plant, usually by a vector (wind, insects or human. Hybrid seeds are usually produced by crossing a female parental line with a male parental line. In order to ensure 100% F_1 hybrid seeds, the line being the female must be 100% male sterile, otherwise self-pollination may occur, producing seeds that will contaminate the F_1 hybrid seeds. Hand or mechanical emasculation of the female line is often done, however, this is an expensive procedure.

Only few commercial hybrid seed systems are currently known for field crops and all of them are based on cytoplasmic male sterile parental lines being the female parent (A-line) of the F_1 hybrid seeds. For example see Frankel R. and Galun, *Pollination mechanisms, reproduction and plant breeding*, Springer, Berlin Heidelberg New York (1977), p. 281 and Kaul M.L. *Male sterility in plants* Springer Verlag, Berlin Heidelberg New York (1978), p. 1005.

There is no conventional way to produce 100% male sterile (female) parents by genetic means that are based on a single recessive gene. In conventional systems which are based on genic male sterility, the male sterility is not 100% and laborious efforts are needed in order to sort out (roguing) the fertile plants in order to produce a population of 100% female plants (male sterile). Therefore, the suggested system according to the present invention is based on genic male sterility that could be regulated to allow transient male fertility restoration (T-MFR) by manipulation of gene expression.

The present invention relates to a method for restoring transient male fertility (T-MFR) in male sterile plants in order to produce 100% male sterile plants suitable for the production of F_1 hybrid seeds which are of commercial value. Said 100% male sterile plant population is achieved by self or cross pollination among the male sterile plants that undergo T-MFR according to the method of the present invention and produce pollen, or by crossing the T-MFR pollen producing plants with male sterile plants.

Such a system allows the propagation of the female line by the T-MFR technique and enables production of seeds that produce 100% male sterile

plants. In other words, the present invention assures genotypes which are 100% male sterile at the seed stage. The plants that develop from these seeds serve as the female line (A-line) for the production of hybrid seeds with the proper male pollinator that is assigned by the breeder (a C line).

It is the aim of the present invention to provide a method for a transient male fertility restoration in plants in order to produce 100% male sterile plants that can be used as the female parents in the hybrid seed production, thus eliminating the self-pollination in the hybrid seed production (as potential contaminants) or the need for roguing and providing a method for low cost production of hybrid seeds.

The present invention also enables 100% permanent male fertility restoration to the F_1 hybrid (commercial) plants. This is a prerequisite for F_1 plants for most of the major crops, in which the economical yield consists of the plant reproductive part. In many crop species (rice, wheat etc.) such technique may be the only way to produce F_1 hybrid seeds using nuclear genes for male sterility and not by resorting to transgenic plants.

SUMMARY OF THE INVENTION

The present invention relates to a method for obtaining seeds that yield 100% male sterile plants to be used as the A-line for hybrid seeds production, comprising; a) selecting male sterile plants; b) inducing transient male fertility restoration (T-MFR) in said plants; c) self-pollination or crossing among plants that undergo T-MFR in step b and produced pollen, or

cross-pollination between pollen produced in step b and any suitable male sterile plants; d) collecting the seeds of the fruits developed by the pollination of step c, said seeds will yield 100% male sterile plants (all female parent line).

According to the present invention T-MFR is produced by subjecting the male sterile plants to controlled conditions of daytime and nighttime temperatures for a predetermined period of time;

According to the present invention the pollination of step (c) can be done between plants in which T-MFR was induced in step (b), being the male parent, and plants in which T-MFR was not induced under the same conditions, being the female parent.

The present invention further relates to the pollen produced in step (b), to the seeds obtained by the method of the present invention that yield 100% male sterile plants, to the 100% male sterile plants developed thereof, to the hybrid seeds that yield F1 male fertile plants produced by the crossing between said 100% male sterile plants as A-line and any other suitable male fertile C-line of homozygous MS·MS allele and to the F1 male fertile plants produced thereof.

The present invention specifically relates to said methods, pollen, seeds and plants of the solanaceae family such as tomato or petunia of any male sterility genes which are of horticultural value and more specifically to tomato plants having the male sterility inducing alleles in homozygous state such as ms26 ms33 or ms35 genome. However similar method can be applied to any species having male sterility inducing gene that can be manipulated.

In a preferred embodiment of the present invention the male sterile plants are tomato plants of the female parent of varieties, such as Orit and Naama, having a male sterility gene of tomato ms26 and the controlled temperature conditions to induce T-MFR are: a) 1 to 5 weeks of daytime temperature of about 15-21°C and nighttime temperature lower than of 10°C, followed by b) approximately 2 weeks of daytime temperature of approximately 26°C and nighttime temperature of approximately 16°C.

Yet, in another preferred embodiment of the present invention the male sterile plants are tomato plants of the female parent of varieties, such as Daniella, having a male sterility gene of tomato ms35 and the controlled temperature conditions to induce T-MFR are: a) 1 to 5 weeks of daytime temperature of about 30°C and nighttime temperature of about 21°C followed by b) approximately 2 weeks of daytime temperature of about 26°C and nighttime temperature of about 17°C.

DETAILED DESCRIPTION OF THE INVENTION

Scheme A on page 8 describes the overall general protocol for obtaining F_1 hybrid seeds according to the method of the present invention. The method is based on pollinating stable (msms) male sterile plants by pollen of male sterile plants (msms) that undergo transient male fertility restoration treatment to obtain seeds which will yield 100% male sterile plants. The so obtained 100% male sterile plants are the female line (A-line) of the hybrid seeds. The hybrid seeds obtained by crossing between said A-line and any suitable C-line yield normal and fertile F_1 plants which show no signs of the T-MFR treatment.

Experiments were carried out with several non-commercial and with commercially available female lines which are based on one recessive male sterility gene.

SCHEME A

A METHOD FOR OBTAINING HYBRID SEEDS ACCORDING TO THE PRESENT INVENTION

SELECTING MALE STERILE PLANTS of msms GENOME

↓

T-MFR TREATMENT

↓

COLLECTING POLLEN OF ms GENOME OF TRANSIENT FERTILE PLANTS

↓

CROSSING BETWEEN POLLEN (ms) and STABLE MALE STERILE PLANTS (msms)

↓

FRUITS WITH SEEDS.
THE SEEDS DEVELOP INTO:

↓

100% MALE STERILE PLANTS of msms GENOME
ALL FEMALE LINE **A – line** FOR HYBRID SEEDS

↓

PRODUCTION OF HYBRID SEEDS BY CROSSING WITH C-LINE POLLEN

↓

NORMAL HYBRID SEEDS
F_1

SCHEME B

A METHOD FOR OBTAINING SEEDS THAT WILL YIELD 100% MALE STERILE PLANTS BY TRANSIENT MALE FERTILITY RESTORATION (T-MFR) IN TOMATO PLANTS HAVING ms26 GENE

SELECTING MALE STERILE PLANTS of ms26ms26

↓

T-MFR TREATMENT
1 – 5 WEEKS OF:
DAYTIME TEMPERATURE 15-21°C
NIGHTTIME TEMERATURE < 10°C

↓

RETURNING PLANTS BACK TO STANDARD GROWING TEMPERATURES

↓

ABOUT 3 WEEKS AFTER THE TERMINATION OF THE T-MFR TREATMENT, FLOWERING AND POLLEN APPEARANCE ARE IN THEIR PEAK

↓

COLLECTING POLLEN WITH ms26 GENE

↓

POLLINATING STABLE MALE STERILE PLANTS (ms26ms26) WITH COLLECTED (ms26) POLLEN

↓

FRUITS WITH

100% ms26ms26 SEEDS

The following examples demonstrate the method of the present invention in tomato plants.

EXAMPLE 1: T-MFR, production of male sterile seeds and production of hybrid seeds in tomatoes of ms26 genome

Scheme B on page 9 describes the protocol for the manipulation of the ms26 male sterility gene in tomatoes plants in order to obtain a 100% male sterile seeds.

Experiments were carried out with several commercialy available female lines which are based on the male sterility gene ms26. The flowers of ms26ms26 genotype have certain morphology (distorted anthers with no fertile pollen) that distinguishes them clearly from the male fertile flowers.

The procedure was carried out by the following steps:

1. Seeds of the female commercial line **1613** were sown in trays.
2. 50% of the population of the plants grown in step 1 were MS26ms26 fertile plants and were eliminated (roguing process) in order to obtain population of 100% male sterile plants with the ms26ms26 genotype only.
3. **T-MFR induction process**: The selected plants of step 2 were transferred into a phytotron. The specific conditions to which the plants were subjected, were normally the following temperature conditions:

   a. 1 to 5 weeks of subjecting the plants to daytime temperature of about 17 ± 3°C and nighttime temperature of about 10 ± 3°C.

b. About 3 weeks of the standard growing conditions for tomatoes 20-29°C/14-18°C.

4. Following the induction of T-MFR, different routes are taken for the production of the pollen and for the growing of the female parent, both for the production of the 100% male sterile plants:

A: Production of pollen

About 3 weeks after the T-MFR treatment (step 3a) terminates, pollen grains develop in the anthers of about 90-100% of the plants which underwent T-MFR. The pollen grains from these plants are collected by any known method including the industrial method using liquid and is used immediately or is stored for a later use. The pollen grains collection lasts as long as they are produced on the T-MFR plants, for about 1 to 2 weeks, depending on the T-MFR length of the induction period.

B: Growing the selected population for the female parent

Those plants that do not show signs of T-MFR during step 3b (after exposure for one week only) can be selected as mother plants at this stage and are planted in a greenhouse or in the field to be the female parents of the female A-line consisting of 100% male sterile plants. Another way of selecting the female plants for A-line is to keep plants under T-MFR induction conditions only for one week and then at the normal growing conditions for tomatoes. Those plants which do not show signs of T-MFR at all are good female plants for A-line.

5. The plants of step 4B (female parent) are pollinated by the pollen collected at step 4A to obtain seeds of 100% male sterility (to develop the A-line plants of the hybrid seeds).

RESULTS

1. Fertility restoration

The effect of the T-MFR is measured by 2 parameters: 1) the number of the plants that respond to the treatment; 2) the number of fertile flowers per plant. The production of these two parameters determines the yield of pollen that can be obtained.

Sorting of plants according to their restored fertility was done according to the following definitions:

S – male sterile without traces of pollen;

N.F – no flowers;

F5 – traces of pollen;

F4 – very little pollen;

F3 – medium amount of pollen as compared to fertile plants. This is usually the stage at which the pollen is collected from the T-MFR plants;

F2 and F1 are definition for a large amount of pollen or full fertile plants, respectively. Such fertility degrees are usually not achieved in the T-MFR plants.

Table 1 shows typical results of different T-MFR induction periods.

Table 1: The degrees of fertility distribution of tomato plants (ms26ms26) after T-MFR treatment:

Induction period	Sorting date	Population distribution					Total no. of plants
		S	F3	F4	F5	N.F.	
15/12/97-30/12/97	30/12/97	273	9	0	0		282
	4/1/97	116	22	47	57		242
10/3/98-25/3/98	10/3/98	78	27			8	123
	30/3/98	92	6	14			112
	8/4/98	109		1			110
	16/4/98	15	85	13			113
31/12/97-2/2/98	28/1/98	62	27	17	26		132
	17/2/98	58	5	8	4		75
	25/2/98	22	84	11			117
	3/3/98	47	40	1	17		105

Table 2 shows typical results of the average percentage of fertile flowers per plant (F3 - fertility level - flowers that are suitable for collecting pollen) after 2 and 3 weeks of T-MFR induction periods, at different times after the T-MFR treatment terminates. The test was carried out by picking an arbitrary sample of plants.

Table 2:Average percentage of fertile flowers per plant after T-MFR treatment.

T-MFR induction period	Date of sampling	Number of sampled plants	Average percentage of fertile flowers per plant (%)
2 weeks 10.3.98 – 25.3.98	21.4.98	12	31.18
3 weeks 4.5.98 – 25.5.98	14.6.98	12	60.41
"	15.6.98	30	39.39
"	16.6.98	24	31.35

2. Seeds that yield A-line plants and hybrid seeds production

Cross and self pollination was carried out among different plants that underwent T-MFR treatment. The seeds so obtained were sown and the plants developed from these seeds were planted in fields or greenhouses at different locations to serve as the A-line of the hybrid seeds and were pollinated by C-line pollen under different conditions.

The load of the seed bearing fruits on a plant is an important factor that affects the hybrid seeds yield. Experiments were done in order to find what is the earliest ripening level at which the hybrid seeds can be collected. The results are summarized in Table 3.

Table 3: The average sprouting rate of the seeds collected at different ripening levels

Fruit ripening level	The average sprouting rate of the different ripening levels			Percentage of sprouting After 8 days (%)		
	After 5 days	After 8 days	After 13 days	Normal sprouts	Abnormal sprouts	No sprouts
Green	25.16	27.5	27.8	27.84	2.33	69.83
Color break	61.2	64.2	64.3	64.35	2	33.65
Orange	80	86		86	2	12
Red	84.3	90.6		90.6	3.05	6.35
Deep red	76.5	90.6		90.6	3.2	6.2

The above results show that the seeds can be collected at the orange stage, 4-6 days before full ripening, thus reducing the load of fruits per plant. Early harvest of the fruits allow ample flowering on the plant and thus increasing the number of fruits per plant during the season.

3. Hybrid plants

Many thousands F_1 hybrid plants of the variety Orit were produced by the hybrid seeds obtained as described above and were compared to plants that were produced by the conventional procedure, by all the morphological parameters. There was no difference between the two groups of plants.

EXAMPLE B: T-MFR, production of male sterile seeds and production of hybrid seeds in tomatoes of ms35 gemome

Similar experiments have been carried out with tomatoes of ms35 genome. Induction period of 10 days with conditions of about 30°C daytime temperature and 21°C nighttime temperature, transient male fertility has been restored in the plants.

CLAIMS

1) A method for obtaining seeds that yield all female 100% male sterile plants for use as the A-line for hybrid seeds production, comprising;

a) selecting male sterile plants;

b) inducing transient male fertility restoration (T-MFR) in said plants ;

c) self-pollination or cross-pollination among plants that undergo T-MFR in step b and produced pollen, or cross-pollination between pollen produced in step b and any suitable male sterile plants;

d) collecting the seeds of the fruits developed by the pollination of step c, said seeds will yield 100% male sterile plants.

2) A method according to claim 1 wherein the transient male fertility restoration is induced by subjecting the male sterile plants to controlled conditions of daytime and nighttime temperatures for a predetermined period of time.

3) A method according to claim 1 wherein the pollination of step c is done between plants in which T-MFR was induced by step b, being the male parent, and plants in which T-MFR was not induced under the same conditions, being the female parent.

4) A method according to claim 1 and 2 wherein the plants are of the solanaceae family.

5) A method according to claim 3 wherein the plants are selected from tomato or petunia.

6) A method according to claim 4 wherein the male sterile plants are tomato plants and wherein the male sterility is of the type containing the male sterility genes ms26ms26 or ms33ms33 or ms35ms35 or any other tomato male sterility genes.

7) A method according to claim 5 wherein the plants are tomato plants having a male sterility gene ms26 and wherein the controlled temperature conditions are as follows:

a) 1 to 5 weeks of daytime temperatures of about 15-21°C and nighttime temperatures lower than 10°C;

followed by

b) approximately 2 to 4 weeks of daytime temperatures of approximately 26°C and nighttime temperatures of approximately 16°C.

8) A method according to claim 5 wherein the plants are tomato plants having a male sterility gene ms35 and wherein the controlled temperature conditions are as follows:

a) 1 to 5 weeks of daytime temperatures of about 30°C and nighttime temperatures lower than 21°C;

followed by

b) approximately 2 to 4 weeks of daytime temperatures of approximately 26°C and nighttime temperatures of approximately 17°C.

9) The pollen produced in step b of claim 1.

10) The seeds that yield 100% male sterile plants, obtained according to the method of claims 1 to 8.

11) The 100% male sterile plants developed from the seeds of claim 10.

12) The hybrid seeds that yield F1 male fertile plants and are produced by crossing between the plants of claim 11, being the female A-line and any other suitable male fertile C-line of homozygous MS·MS allele.

13) The F1 male fertile plants as defined in claim 12.

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/IL 00/00436

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A01H1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1997 MIKESELL JAN E: "Influences on the life cycle in Pharbitis nil Choisy (Convolvulaceae) induced by low temperature." Database accession no. PREV199799664193 XP002152410 abstract & INTERNATIONAL JOURNAL OF PLANT SCIENCES, vol. 158, no. 3, 1997, pages 306-312, ISSN: 1058-5893 -/--	1,9-13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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